Amendment

In the Specification

Please replace the paragraph on page 1, above the heading "Background of the Invention" with the following paragraph.

This application is a divisional of U.S.S.N. 09/763,620 filed March 2, 2001, now U.S. Patent No. 6,936,425 issued August 30, 2005, entitled "*Attenuated Salmonella SP12 Mutants as Antigen Carriers*", which is a filing under 35 USC §371 of PCT/EP99/06514 (WO 00/14240) filed on September 3, 1999, which claims priority to European Patent Application No. 98116827.1 filed September 4, 1998.

Please replace the paragraph on page 41, lines 12-24 with the following paragraph.

No expression of *sseB* or *sscA* was observed during growth in various rich media, or cell culture media with or without serum. However, low amount of SsaP were detected after growth in ~~LB~~ Lysogeny Broth (LB) or other rich media such as brain ~~hart~~ heart infusion (BHI). Growth in minimal medium containing less than 30 μM Mg^{2+} induces the expression of SPI2 genes. Such effect of the Mg^{2+} concentration has so far only been observed for PhoP/PhoQ-regulated genes. This observation is in contrast to a previous report by Valdivia and Falkow (1997) who postulated that SPI2 gene expression is independent of PhoP/PhoQ. However, in a $PhoP^c$ (constitutive) strain background (CSO22, Miller *et al.*, 1989) expression of SPI2 genes was not constitutive but still dependent on the Mg^{2+} concentration of the medium. This indicates that SPI2 gene

expression is modulated by PhoP/PhoQ, but that further regulatory elements such as SsrA/B are needed.

Please replace the paragraph spanning pages 47 and 48 with the following paragraph.

A deletion of 158 bp between codon 264 and 422 of *sseC* was generated. Plasmid p5-2 was digested by *Cla*I and the larger fragment containing the vector portion was recovered and self-ligated to generate p5-60. Plasmid p5-60 was linearized by digestion with *HindIII*, which cuts once within the *sseC* gene. Primers *sseC*-del-1 (5'-GCT AAG CTT CGG CTC AAA TTG TTT GGA AAA C-3') (SEQ ID NO:52) and *sseE*-del-2 (5'-GCT AAG CTT AGA GAT GTA TTA GAT ACC-3') (SEQ ID NO:53) were designed to introduce *HindIII* sites. PCR was performed using linearized p5-60 as template DNA. The ~~TaqPlus~~ TAQPLUS® polymerase (*Pyrococcus furiosus* DNA polymerase + recombinant *T. Aquaticus YT1* polymerase) (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x ~~TaqPlus~~ TAQPLUS® Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p5-8), 250 ng of each primer and 5 U of ~~TaqPlus~~ TAQPLUS® DNA polymerase (*Pyrococcus furiosus* DNA polymerase + recombinant *T. Aquaticus YT1* polymerase). PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *HindIII*, self-ligated, and the ligation mixture was used to transform *E. coli* DH5α to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction

digestion and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *XbaI* and *KpnI* and ligated to *XbaI/KpnI*-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λpir to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (NaI^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 μg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseC* locus was performed by PCR using primers *sseC*-For (5'-ATT GGA TCC GCA AGC GTC CAG AA-3') (SEQ ID NO: 54) and *sseC*-Rev (5-TAT GGA TCC TCA GAT TAA GCG CG-3') (SEQ ID NO: 55). Amplification of DNA from clones containing the wild-type *sseC* allele resulted in a PCR product of 1520 bp, use of DNA from clones harbouring a *sseC* allele with an internal deletion resulted in a PCR product of 1050 bp. The integrity of clones harbouring the *sseC* deletion was further confirmed by Southern analysis of the sseC locus. Finally, the *sseC* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy et al., 1996) and the resulting strain was designated MvP 337.

Please replace the paragraph spanning pages 48 and 49 with the following paragraph.

A deletion of 116 bp between codon 26 and 142 of *sseD* was generated. Plasmid p5-2 was digested by *Hin*dIII/*Pst*I and a fragment of 2.1kb was isolated and subcloned in *Hin*dIII/*Pst*I-digested vector pBluescript SK+. The resulting construct was designated p5-8. p5-8 was linearized by digestion with *Eco*RV, which cuts twice within the *sseD* gene. Primers *sseD*-del-1 (5'- ATA GAA TTC GGA GGG AGA TGG AGT GGA AG -3') (SEQ ID NO:56) and *sseD*-del-2 (5'- ATA GAA TTC GAA GAT AAA GCG ATT GCC GAC -3') (SEQ ID NO:57) were designed to introduce *Eco*RI sites. PCR was performed using linearized p5-8 as template DNA. The ~~TaqPlus~~ TAQPLUS® polymerase (*Pyrococcus furiosus* DNA polymerase + recombinant *T. Aquaticus YT1* polymerase) (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x ~~TaqPlus~~ TAQPLUS® Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p5-8), 250 ng of each primer and 5 U of ~~TaqPlus~~ TAQPLUS® DNA polymerase (*Pyrococcus furiosus* DNA polymerase + recombinant *T. Aquaticus YT1* polymerase). PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 5 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Eco*RI, self-ligated, and the ligation mixture was used to transform *E. coli* DH5á to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction mapping and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λ*pir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed

according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 ig/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseD* locus was performed by PCR using primers *sseD*-For (5'- GAA GGA TCC ACT CCA TCT CCC TC -3') (SEQ ID NO:58) and *sseD*-Rev (5- GAA GGA TCC ATT TGC TCT ATT TCT TGC-3') (SEQ ID NO:59). Amplification of DNA from clones containing the wild-type *sseD* allele resulted in a PCR product of 560 bp, use of DNA from clones harbouring a *sseD* allele with an internal deletion resulted in a PCR product of 220 bp. The integrity of clones harbouring the *sseD* deletion was further confirmed by Southernanalysis of the *sseD* locus. Finally, the *sseD* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP338.

Please replace the paragraph spanning pages 50 and 51 with the following paragraph.

A deletion of 128 bp between codon 32 and 160 of *ssc*B was generated. A 3kb *Bgl*II fragment of plasmid p5-2 was ligated into the *Bam*HI site of pBluescript KS+ to generate plasmid p5-70. Plasmid p5-70 was linearized by digestion with *Nco*I, which cuts once within the *sscB* gene. Primers *sscB*-del-1 (5'- ATG GGA TCC GAG ATT CGC CAG AAT GCG CAA -3') (SEQ ID NO:60) and *sscB*-del-2 (5'- ATG GGA TCC ACT GGC ATA

AAC GGT TTC CGG -3') (SEQ ID NO:61) were designed to introduce *Bam*HI sites. PCR was performed using linearized p5-70 as template DNA. The ~~TaqPlus~~ TAQPLUS® polymerase (*Pyrococcus furiosus* DNA polymerase + recombinant *T. Aquaticus YT1* polymerase) (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x ~~TaqPlus~~ TAQPLUS® Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p5-70), 250 ng of each primer and 5 U of ~~TaqPlus~~ TAQPLUS® DNA polymerase (*Pyrococcus furiosus* DNA polymerase + recombinant *T. Aquaticus YT1* polymerase). PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Bam*HI, self-ligated, and the ligation mixture was used to transform *E. coli* DH5á to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction analysis and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λ*pir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 ìg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to

streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *sseC* locus was performed by PCR using primers *sscB*-For (5'- ATT GGA TCC TGA CGT AAA TCA TTA TCA -3') (SEQ ID NO:62) and *sscB*-Rev (5- ATT GGA TCC TTA AGC AAT AAG TGA ATC -3') (SEQ ID NO:63). Amplification of DNA from clones containing the wild-type *sscB* allele resulted in a PCR product of 480 bp, use of DNA from clones harbouring a *sscB* allele with an internal deletion resulted in a PCR product of 100 bp. The integrity of clones harbouring the *sseC* deletion was further confirmed by Southernanalysis of the *sscB* locus. Finally, the *sscB* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP339.

Please replace the paragraph on page 64, lines 6-12 with the following paragraph.

FACSCAN™ (cytometer) Analysis

Approximately $5x10^5$ cells were incubated in staining buffer (PBS supplemented with 2% FCS and 0.1% sodium azide) with the desired antibody or combination of antibodies for 30 min at 4°C. After washes, cells were analysed on a ~~FACScan~~ FACSCAN™ (cytometer) (Becton Dickinson). The monoclonal antibodies used were FITC-conjugated anti-CD4 and anti-CD8 (clones H129.19 and 53-6.7; Pharmingen).

Please replace the paragraph spanning pages 63 and 64 with the following paragraph.

The CD8+ cell subset was depleted using ~~MiniMACS~~ MINIMACS™ (cell separator) Magnetic Ly-2 Microbeads according to the manufacturer's instructions (Miltenyi Biotec). Depleted cell preparations contained 1% CD8+ cells.

Please replace the paragraph on page 64, lines 20-31 with the following paragraph.

Spleen cell suspensions were enriched for CD4+ T cells using ~~MiniMACS~~ MINIMACS™ (cell separator) Magnetic Ly-2 and indirect goat-anti-mouse-IgG Microbeads according to the instructions of the manufacturer (Mitenyi Biotec GmbH, Germany). Cell preparations contained >65% of CD4+ cells. Cells were adjusted to $2x10^6$ cells/ml in complete medium supplemented with 20 U/ml of mouse rIL-2 (Pharmigen), seeded at 100 µl/well in a flat-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark) and incubated for four days in the presence of different concentrations of soluble 1-gal. During the final 18 hours of culture 1 µCi of [^{3}H]-thymidine (Amersham International, Amersham, U.K.) was added per well. The cells were harvested on paper filters using a cell harvester and the [^{3}H]-thymidine incorporated into the DNA of proliferating cells was determined in a .beta.-scintillation counter.

Please replace the paragraph spanning pages 74 and 75 with the following paragraph.

A deletion of 407 codons between codon 44 and 451 of *ssrB* was generated. Plasmid p2-2 was digested by *Bam*HI and *Kpn*I, a fragment of 3.7kb was recovered and subcloned in pBluescript KS+ to generate p2-50. Plasmid p2-50 was linearized by digestion with *Pst*I, which cuts once within the subcloned fragment of the *ssrA* gene. Primers *ssrA*-del-1 (5'- GGT CTG CAG GAT TTT TCA CGC ATC GCG TC -3') (SEQ ID NO:68) and *ssrB*-del-2 (5'- GGT CTG CAG AAC CAT TGA TAT ATA AGC TGC -3') (SEQ ID NO:69) were designed to introduce *Pst*I sites. PCR was performed using linearized p2-50 as template DNA. The ~~TaqPlus~~ TAQPLUS® polymerase (*Pyrococcus furiosus* DNA

polymerase + recombinant *T. Aquaticus YT1* polymerase) (Stratagene) was used according to the instructions of the manufacturer. Reactions of 100 µl volume were set up using 10 µl of 10 x ~~TaqPlus~~ TAQPLUS® Precision buffer containing magnesium chloride, 0.8 µl of 100 mM dNTPs, 250 ng DNA template (linearized p2-50), 250 ng of each primer and 5 U of ~~TaqPlus~~ TAQPLUS® DNA polymerase (*Pyrococcus furiosus* DNA polymerase + recombinant *T. Aquaticus YT1* polymerase). PCR was carried out for 35 cycles of: 95°C for 1 minute, 60°C for 1 minute, 72°C for 6 minutes. Then a final step of 72°C for 10 minutes was added. 10 µl of the PCR reaction were analyzed. A product of the expected size was recovered, digested by *Pst*I, self-ligated, and the ligation mixture was used to transform *E. coli* DH5á to resistance to carbenicillin. Plasmids were isolated from transformants and the integrity of the insert and the deletion was analyzed by restriction analysis and DNA sequencing. The insert of a confirmed construct was isolated after digestion with *Xba*I and *Kpn*I and ligated to *Xba*I/*Kpn*I-digested vector pKAS32. The resulting construct was used to transform *E. coli* S17-1 λ*pir* to resistance to carbenicillin, and conjugational transfer of the plasmid to *S. typhimurium* (Nal^R, $Strep^R$) was performed according to standard procedures (de Lorenzo and Timmis, 1994). Exconjugants that had integrated the suicide plasmid by homologous recombination were selected by resistance to nalidixic acid and carbenicillin, and screened for sensitivity to streptomycin. Such clones were grown in LB to OD600 of about 0.5 and aliquots were plated on LB containing 250 µg/ml streptomycin to select for colonies which had lost the integrated plasmid and undergone allelic exchange. Clones resistant to streptomycin but sensitive to carbenicillin were used for further analysis. Screening of mutants with a deletion within the *ssrA* locus was performed by PCR using primers *ssrA*-For (5'- AAG GAA TTC AAC AGG CAA CTG

GAG G-3') (SEQ ID NO:64) and *ssrA*-Rev (5- CTG CCC TCG CGA AAA TTA AGA TAA TA -3') (SEQ ID NO:65). Amplification of DNA from clones containing the wild-type *ssrA* allele resulted in a PCR product of 2800 bp, use of DNA from clones harbouring a *ssrA* allele with an internal deletion resulted in a PCR product of 1580 bp. The integrity of clones harbouring the *ssrA* deletion was further confirmed by Southern analysis of the *ssrA* locus. Finally, the *ssrA* locus containing the internal in-frame deletion was moved into a fresh strain background of *S. typhimurium* by P22 transduction (Maloy *et al.*, 1996) and the resulting strain was designated MvP340.